THE CALCIUM CONTENT OF LEGUME ROOT NODULES.

By H. L. Jensen, Macleay Bacteriologist to the Society.
(*From the Department of Bacteriology, University of Sydney.*)

[Read 28th May, 1947.]

Introduction.

It is well known that the dry matter of leguminous plants is generally richer in calcium than that of non-legumes, and this has sometimes been taken as indicating that calcium is of specific importance for the process of symbiotic nitrogen fixation in the legumes, as well as for the non-symbiotic fixation by *Azotobacter*. Wilson (1940) has pointed out, however, that this high calcium content is also found in leguminous plants grown with combined nitrogen, a fact which suggests that the general metabolism of the legumes requires more calcium than that of other plants. Most of the available analytical figures refer to the aerial parts (stems and leaves) of legumes; there are few data concerning the roots, and the calcium content of the root nodules—the actual substrate of the process of nitrogen fixation—seems to have been determined quantitatively only in the more than sixty-year-old analyses of Troschke (1884, quoted by Czapek (1920) and Fred *et al.* (1932)), who found that the dry matter of roots and root nodules of lupins grown in water culture contained 0·46 and 0·75% of calcium,* respectively; the proportion of calcium in the ash of the two materials was not much different. Konishi and Tsuge (1936) made spectrographic determinations which were little more than qualitative, but which in most cases indicate a higher concentration of calcium in the nodules than in the roots of various legumes at successive stages of growth.

A number of pot experiments with lucerne and subterranean clover, of which a preliminary account has been given elsewhere (Jensen, 1946), yielded several samples of top, root and nodule materials which were analysed for calcium in order to find a possible clue to the question whether or not this element performs a specific function in the process of symbiotic nitrogen fixation as well as in *Azotobacter* (Burk and Lineweaver, 1931; Horner and Burk, 1934). Materials from a single experiment with field peas were also included.

Experimental.

Calcium was determined by Shapter's method as described by Piper (1942), with some minor modifications that were rendered necessary by the fact that only small amounts of nodule-substance, mostly 0·2–0·4 gm., were available for analysis. The volume of liquid in which the precipitation of calcium oxalate took place was reduced to about 40 ml., the precipitate was collected and washed by centrifugation instead of by filtration, and 0·05N potassium permanganate (1 ml. = 1 mgm. Ca) was used for the titration. In most cases there was not sufficient nodule-substance for duplicate determinations, but it was found that small known quantities of calcium could be recovered with a high degree of accuracy, as shown by the following figures:

Mgm. Ca taken	1·0	2·0	5·0	10·0
„ „ recovered—				
a.	1·02	2·05	4·96	9·95
b.	1·04	2·06	5·00	10·15

Firstly, a set of calcium determinations was made in nodules of subterranean clover and roots and nodules of lucerne, grown for 15 and 20 weeks, respectively, in acid sand adjusted to four ranges of reaction by addition of increasing amounts of calcium carbonate, besides a basal fertilizer-mixture containing adequate amounts of

* Or calcium oxide? The same figures are given by Fred *et al.* (1932) as "calcium" and by Czapek (1920) as "Kalk" (presumably CaO). Troschke's original paper has not been available to the author.

calcium as $CaSO_4$. The nodule-tissue in both plants showed a high degree of efficiency, fixing during the whole growth-period from 1,900 to 3,500 mgm. nitrogen per gm. dry nodule-substance in lucerne, and 720 to 1,200 mgm. in clover. The results are seen in Table 1, which shows that in both plants the calcium content of the nodules is of an order similar to that found by Troschke, and that there is no evidence of a clear-cut effect of the reaction. The lucerne roots are seen to contain only about one-half to one-third as much calcium as the nodules on the basis of dry matter, but the content of calcium in proportion to nitrogen is approximately the same in both plant organs, and actually higher in roots, at pH 7·6–7·7.

Another set of calcium determinations was made on top, root and nodule material of lucerne ("Giant Upright"), subterranean clover ("Mount Barker" and "Dwalganup"), and field peas. The source of nitrogen was atmospheric nitrogen unless otherwise stated. The results are seen in Table 2.

TABLE 1.

Calcium Content of Root Nodules of Lucerne and Subterranean Clover, and of Roots of Lucerne, grown in Sand of Different Reaction.

	% $CaCO_3$ added to sand	0	0·01	0·04	0·4
Lucerne.	pH of sand initially	4·7–4·9	5·4–5·5	6·3–6·6	7·0–7·1
	,, ,, ,, finally	4·3–4·5	4·7–4·1	6·9–7·2	7·6–7·7
	% Ca in nodules (dry matter)	0·95	0·73*	0·63	0·76
	,, ,, ,, roots ,, ,, ...	0·27	0·27	0·24	0·37
	Ratio N/Ca in nodules	7·7	9·6	11·4	9·0
	,, ,, ,, roots	8·6	9·2	9·9	5·1
Clover.	pH of sand initially	4·5	4·8–4·9	6·0–6·1	6·7
	,, ,, ,, finally	4·1–4·3	4·5–4·7	6·2–6·4	7·6–7·7
	% Ca in nodules (dry matter)	0·46	0·58	0·75	0·55

* This figure is of doubtful accuracy because only a very small amount of substance was available for analysis.

TABLE 2.

Calcium Content and Ratio of Nitrogen to Calcium in Tops, Roots and Root Nodules of Leguminous Plants grown in Sand Culture.

Plant Species and Age.	Final pH of Sand.	% Ca in Dry Matter.			Ratio N/Ca.		
		Tops.	Roots.	Nodules.	Tops.	Roots.	Nodules.
1. Lucerne, 105 days ..	4·9–5·4	1·35	0·19	0·47	2·16	10·6	14·6
2. Lucerne, 88 days	5·1–5·6			0·55			13·1
3. Same, 102 days	6·0–6·1	1·49	0·24	0·35	2·11	10·2	20·5
4. Same, sand + $CaCO_3$, 88 d.	7·3–7·5			0·64			12·7
5. Same, 102 days	7·0–7·5	1·88	0·40	0·76	1·58	4·97	9·1
6. Sub. clover, 130 days ..	4·9–5·0	1·51	0·47	0·41	2·19	5·60	16·1
7. Same, combined nitrogen ($NaNO_3$ + $(NH_4)_2SO_4$), 130 d.	4·8–5·3	1·18	0·43	0·34	2·48	6·49	19·3
8. Same, free N, sand + $CaCO_3$, 130 days	7·2–7·3	1·90		0·52	1·52		12·6
9. Sub. clover, 96 days ..	4·3–4·7	2·19	0·52	0·51	1·15	4·51	12·2
10. Same, combined nitrogen (NH_4NO_3), 96 days ..	4·7–4·8	1·96	0·54	0·41	1·39	5·16	15·1
11. Same, sand + $CaCO_3$, free nitrogen, 96 days	7·1–7·2	2·48	0·73	0·56	0·98	3·00	11·6
12. Same, combined nitrogen (NH_4NO_3), 96 days ..	7·3–7·5	2·63	0·84	0·54	1·02	3·17	13·3
13. Sub. clover ("Dwalganup"), 105 days	7·0–7·5	1·22	0·51	0·37	2·13	4·86	19·8
14. Field peas, 83 days ..	7·3–7·7	0·81	0·98	0·51	3·78	2·76	11·3

In all three plant species the calcium content of the nodules appears fairly constant and rarely departs much from 0·5%. In parallel experiments with acid and alkaline sand the calcium content is higher at alkaline reaction, but the difference is not very marked except in one case (lucerne after 102 days, Analyses Nos. 3 and 5). It is also noteworthy that provision of combined nitrogen does not cause any marked reduction in the calcium content of the small amount of nodule tissue that develops under these conditions. The calcium content of the clover roots is mostly similar to that of the nodules, but in the lucerne roots it is considerably lower (as in Table 1, and as found by Troschke in lupins). The pea tops appear somewhat low in calcium, but the tops of lucerne and clover show contents of 1·2–2·6% calcium, quite similar to figures quoted by Wilson (1940), slightly lower at acid reaction but not much influenced by the source of nitrogen. The nodules thus appear quite poor in calcium in comparison with the tops, and in proportion to the content of nitrogen the nodules actually contain less calcium than the other parts of the growing plant, as shown by the N/Ca ratios in Table 2.*

These results lend no support to the view that calcium is specifically needed for the process of symbiotic nitrogen fixation. (Neither can any such evidence be drawn from the fact that calcium stimulates nodule formation in soy bean seedlings at pH-values above 5·0, as shown by Albrecht (1933), since the number of nodules formed is not necessarily an index of the resulting nitrogen fixation; in this connection it is interesting to note the recent observation by Anderson and Thomas (1946) that molybdenum increases the nitrogen-fixing activity of the nodule-tissue, while actually reducing the number of nodules, a result which the present writer has been able to confirm.) It is possible, however, that calcium is needed in the tops and roots for other physiological purposes, such as neutralization of organic acids, synthesis of asparagin, activation of proteolytic enzymes, etc. (Nightingale, 1937; Wilson, 1940), in quantities which outweigh those that might be required for the functioning of the nodule-tissue, and this would presumably, like other plant tissues, require certain amounts of calcium apart from its possible importance for the specific process of nitrogen fixation. If calcium in this respect functions as a "trace element", even its concentration in the nodules appears relatively high. Assuming that fresh nodule-substance contains 25% dry matter and has a specific gravity of 1 (actually somewhat higher), a content of 0·5% calcium in dry matter corresponds to a concentration of 0·125 mgm. calcium per cubic centimetre of nodule-tissue, or roughly 3×10^{-3} molar. This is considerably more than the quantities required by *Azotobacter*, in which Burk and Lineweaver (1931) found that a supply of 25–50 mgm. calcium per litre, or $0{\cdot}6$–$1{\cdot}2 \times 10^{-3}$ molar, was necessary for normal growth with free nitrogen, while Burk and Horner (1934) later found that concentrations of $0{\cdot}02$–$0{\cdot}04 \times 10^{-3}$ molar were sufficient for half-optimal rate of fixation (it may be noted, however, that this was probably under conditions of partial molybdenum-deficiency).

Upon the whole it seems that the question of the essentiality of calcium for symbiotic nitrogen fixation cannot be finally answered until it becomes possible to make the root nodule bacteria fix nitrogen *in vitro*.

Summary.

Effective root nodules of lucerne, subterranean clover and field peas, grown in sand culture, contained from 0·34 to 0·95% calcium in dry matter, compared with 0·19 to 0·98% in the roots and 0·8 to 2·6% in the tops. Although usually higher at alkaline reaction, the calcium content of the nodules was mostly not strongly influenced by the reaction of the sand or the supply of combined nitrogen to the plants. The nodules contained less calcium in proportion to nitrogen than did either the roots or

* The seeds from which the plants were grown proved even poorer in calcium, both absolutely and in proportion to nitrogen as shown by the following analytical figures:

	Lucerne.	Sub. Clover. ("Dwalganup").	Pea.
% Ca in dry matter	0·15	0·20	0·14
Ratio N/Ca	40·7	25·5	22·1

the tops. The results give no evidence that calcium is needed specifically for the process of nitrogen fixation, but do not allow any final conclusion in this respect.

References.

ALBRECHT, W. A., 1933.—Inoculation of Legumes as related to Soil Acidity. *J. Amer. Soc. Agron.*, 25 : 512-522.

ANDERSON, A. J., and THOMAS, MARGARET P., 1946.—Plant Responses to Molybdenum as a Fertilizer. 1. Molybdenum and Symbiotic Nitrogen Fixation. *Counc. Sci. Industr. Res.*, Melbourne, Bull. No. 198.

BURK, D., and LINEWEAVER, H., 1931.—The Influence of Calcium and Strontium upon the Catalysis of Nitrogen Fixation by *Azotobacter*. *Arch. Mikrobiol.*, 2 : 155-186.

CZAPEK, F., 1920.—Biochemie der Pflanzen. Vol. 2, IV: Die Mineralstoffe im pflanzlichen Stoffwechsel. Jena.

FRED, E. B., BALDWIN, I. L., and MCCOY, ELIZABETH, 1932.—Root Nodule Bacteria and Leguminous Plants. Madison.

HORNER, K., and BURK, D., 1934.—Magnesium, Calcium and Iron Requirements for Growth of *Azotobacter* in Free and Fixed Nitrogen. *J. Agric. Res.*, 48 : 981-995.

JENSEN, H. L., 1946.—The Nitrogen-fixing Activity of Legume Root Nodules. *Aust. J. Sci.*, 9 : 118.

KONISHI, K., and TSUGE, T., 1936.—On the Mineral Matters of Certain Leguminous Crops. *Mem. Coll. Agric., Kyoto Imp. Univ.*, No. 37.

NIGHTINGALE, G. T., 1937.—The Nitrogen Nutrition of Green Plants. *Bot. Rev.*, 3 : 85-174.

PIPER, C. S., 1942.—Soil and Plant Analysis. Adelaide.

WILSON, P. W., 1940.—The Biochemistry of Symbiotic Nitrogen Fixation. Madison.